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A comparison of the action of the endothelium-derived relaxant factor and the inhibitory factor from the bovine retractor penis on rabbit aortic smooth muscle

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1 The dependence of relaxation of rabbit aortic strips by carbachol and by the inhibitory factor from the bovine retractor penis (BRP) on the presence of endothelium has been compared. Carbachol-induced relaxation is abolished by removing the endothelium, inhibitory factor-induced relaxation is unimpaired. The inhibitory factor, therefore, does not act by releasing an endothelium-derived relaxing factor (EDRF).

2 The effect of inhibitors of eicosanoid metabolism on relaxation was examined. Quinacrine and nordihydroguaiaretic acid abolished the relaxant effect of carbachol and flurbiprofen had no effect. The relaxation produced by the inhibitory factor was unaffected by quinacrine and flurbiprofen while nordihydroguaiaretic acid potentiated the response. No eicosanoid appears, therefore, to be involved in the relaxant effect of the inhibitory factor from the BRP.

3 Methylene blue, a drug reported to inhibit guanylate cyclase, in a concentration of 10 μM selectively abolished the relaxation produced by carbachol. However, at the higher concentration of 30 μM it abolished almost completely the response to inhibitory factor from the BRP and reduced inhibition by sodium nitroprusside.

4 It is not possible from these results to exclude the possibility that the EDRF and the inhibitory factor from the BRP are chemically related.

Introduction

The bovine retractor penis muscle (BRP) receives a motor adrenergic innervation and an inhibitory non-adrenergic non-cholinergic (NANC) innervation (Klinge & Sjöstrand, 1974). One putative transmitter of NANC inhibition of the BRP muscle is the as yet unidentified inhibitory factor first extracted by Ambache *et al.* (1975). Further studies from this laboratory have provided more information about the nature of this inhibitory material extracted from the BRP (Gillespie & Martin, 1978; 1980; Bowman *et al.*, 1979; Gillespie *et al.*, 1981; Bowman & Drummond, 1984; McDonald & Gillespie, 1984), which, as well as mimicking the relaxant response to nerve stimulation, is a powerful vasodilator of numerous isolated blood vessels (Bowman *et al.*, 1981; Bowman & Gillespie, 1983).

Relaxation of arterial smooth muscle by several pharmacological agents, characteristically carbachol, is not due to a direct action on the muscle, but dependent upon the presence of the endothelium (Furchgott & Zawadzki, 1980). It has been proposed that endothelial cells exposed to various agents release a substance which relaxes the smooth muscle. The substance released is thought to be a derivative of arachidonic acid or some other unsaturated fatty acid, since the inhibition of phosphilipase A_2 inhibits the endothelium-derived agonist-induced relaxation (Furchgott & Zawadzki, 1980; Singer & Peach, 1983). Since anoxia and inhibition of lipoxygenase inhibited the relaxation it was suggested that the endothelium-derived relaxing factor (EDRF) is an oxidation product of arachidonic acid; a hydroperoxide or a free radical (Furchgott, 1983; 1984). Subsequent studies have shown that the endothelium-derived agonist-induced relaxation of arterial smooth muscle was linked to the activation of guanylate cyclase (Rapoport & Murad, 1983a, b; Diamond & Chu,

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1983). The inhibitory factor from the BRP may owe its action to the release of this EDRF and the first question the experiments described here were designed to answer was whether this was so, by comparing the effect of removing the endothelium on the inhibitory response to carbachol (endothelium-dependent) and on the inhibitory response to the factor extracted from the BRP. We have also investigated the involvement of fatty acid metabolites and guanylate cyclase activity in the inhibitory response.

Methods

The method of extraction of the inhibitory factor from the BRP has been described previously (Gillespie *et al.*, 1981; Bowman & Gillespie, 1982). Partially purified inhibitory factor was present in freeze dried powder mainly consisting of the sodium chloride used to elute the ion exchange column during purification. This powder was reconstituted immediately before use in distilled water to produce a solution of which 1 ml was equivalent to 1 g wet weight of muscle. On some occasions the freeze dried powder was extracted with 2 ml of methanol to reduce the salt content. After evaporating off the methanol the powder was reconstituted in distilled water to give a solution in which 1 ml was equivalent to 3 g wet weight muscle. Inhibitory activity was induced in these solutions by acidification to pH 2 with 5 N HCl for 10 min followed by neutralization to pH 6.8 with 5 N NaOH. These acid-activated samples were then kept on ice.

Dutch male rabbits (2–2.5 kg) were stunned by a blow to the head and killed by exsanguination. The abdominal aorta was excised, trimmed of fat and connective tissue and paired spiral strips cut with fine scissors under microscope observation. In one strip of each pair the endothelium was removed by gentle rubbing of the intimal surface with filter paper moistened in Krebs solution. Intact strips and strips with disrupted endothelium were suspended in a 10 ml organ bath of Krebs solution at 37°C, gassed with 95% O_2 plus 5% CO_2. Throughout the preparation and mounting of spiral strips, the tissue was kept wet with Krebs solution and special care was taken to avoid unintentional rubbing of the intimal surface. The Krebs solution had the following composition (mM): Na^+ 145, K^+ 5.9, Ca^{2+} 2.5, Mg^{2+} 1.2, Cl^- 127, HCO_3^- 25, $H_2PO_4^-$ 1.2, glucose 11, and contained ascorbic acid (30 μM) and disodium edetate (EDTA) (11.4 μM).

During a 60–90 min equilibration period the strips were stretched to a final resting tension of 1.0 g with Krebs solution changes every 20 min. Care was taken not to overstretch the strips. Contractions were measured isometrically by means of a Grass force transducer (model FT 03) and recorded on a Grass model 7 polygraph. To display inhibitory responses, the arterial strips were preconstricted with noradrenaline in a concentration (300 nM) that resulted in 60–70% of the maximal contraction. On this background of tone the inhibitory responses to cumulative doses of carbachol, inhibitory factor, sodium nitroprusside or caffeine were displayed. The functional integrity of the endothelium in unrubbed strips was confirmed by the inhibitory response to carbachol. In studying the effects of quinacrine, flurbiprofen, nordihydroguaiaretic acid and methylene blue, on these inhibitory responses the tissues were exposed to the drug for 15 min (60 min for methylene blue) then reconstricted with noradrenaline and cumulative dose-response curves to the relaxants repeated.

Noradrenaline, carbachol, caffeine, sodium nitroprusside, flurbiprofen and methylene blue were dissolved in 0.9% w/v NaCl solution and kept on ice during the experiments. Quinacrine was dissolved in distilled water and nordihydroguaiaretic acid dissolved in dimethylsulphoxide (DMSO). The final concentration of DMSO did not exceed 0.1% in the organ bath. This concentration of DMSO tested alone had no significant effect on the contractile response to noradrenaline or the dose-response (relaxation) relationship of carbachol, inhibitory factor, sodium nitroprusside or caffeine.

Statistical significance of differences between corresponding points on the dose-response curves were assessed by Student's *t* test for paired observations. A *P* value of less than 0.05 was considered significant. All values are reported and graphed as means ± s.e.mean.

Drugs

Drugs used were: L-ascorbic acid (BDH); caffeine citrate (BDH); carbachol (carbamylcholine chloride, Sigma); dimethylsulphoxide (DMSO) (Sigma); ethylenediaminetetracetic acid disodium salt (EDTA) (Sigma); flurbiprofen (Boots); methylene blue (T & H Smith Ltd.); (−)-noradrenaline bitartrate (Koch-Light); nordihydroguaiaretic acid (Sigma); quinacrine (Sigma) and sodium nitroprusside (BDH).

Results

Endothelium dependence of inhibitory responses

The effect of removing the endothelium on the inhibitory responses to four stimuli was examined. Carbachol which is known to be endothelium-dependent, sodium nitroprusside which has a direct action on smooth muscle and is endothelium-independent, caffeine which inhibits phosphodiesterase and would also be expected to be endothelium-independent, and,

finally, the inhibitory factor from the BRP. Figure 1 illustrates a representative experiment comparing the effect of each of these stimuli on paired strips one with and one without endothelium. Carbachol, as expected, was a powerful relaxant in the presence of endothelium but in the rubbed strip the response was reversed to a small contraction. This reversal was seen in every experiment and was evidence that the endothelium has been removed. In contrast, removing the endothelium had no effect on the relaxation produced by the inhibitory factor or by caffeine and that to sodium nitroprusside (30 nM) was increased from an average of 64 ± 6% to 90 ± 5% ($n = 5$) of the noradrenaline-induced tension.

Figure 2 shows cumulative dose-response curves for inhibition by two of these stimuli, carbachol and the inhibitory factor. In unrubbed strips in which tone was induced by 300 nM Na, carbachol-induced relaxation had a threshold of about 100 nM and resulted in a maximum of 80% relaxation at 3 μM. In rubbed strips carbachol never produced relaxation but at a higher threshold of 1 μM caused contraction. In rubbed strips, in the absence of noradrenaline-induced tone, carbachol caused contraction at a threshold of about 300 nM, close to that for the inhibitory response in the presence of endothelium. By contrast the relaxation produced by the inhibitory factor was slightly greater (not significant) in the presence of endothelium.

The involvement of fatty acid derivatives in the inhibitory response

Quinacrine which inhibits phospholipase A_2 and nordihydroguaiaretic acid which inhibits lipoxygenase abolish carbachol-induced relaxation of aortic strips (Furchgott, 1983; Singer & Peach, 1983) but flurbiprofen which inhibits cyclo-oxygenase is without effect (Furchgott & Zawadzki, 1980). We have, therefore, examined the effects of these three drugs on the relaxation produced by the inhibitory factor and the results are summarised in Figure 3. Neither quinacrine (10 μM) nor flurbiprofen (15 μM) affected the relaxation to inhibitory factor. Nordihydroguaiaretic acid (25 μM), however, significantly potentiated the relaxant effect of the inhibitory factor. The effects on the response to carbachol of these three drugs at the same concentrations were examined. Both quinacrine and nordihydroguaiaretic acid abolished relaxation, and flurbiprofen was without effect confirming the results of Furchgott (1983) and Singer & Peach (1983). None of these inhibitors influenced the relaxation produced by caffeine.

The potentiation of the relaxant effect of the inhibitory factor by nordihydroguaiaretic acid was unexpected. Relaxation of the bovine retractor penis by inhibitory nerve stimulation or by the inhibitory factor appears to involve activation of guanylate

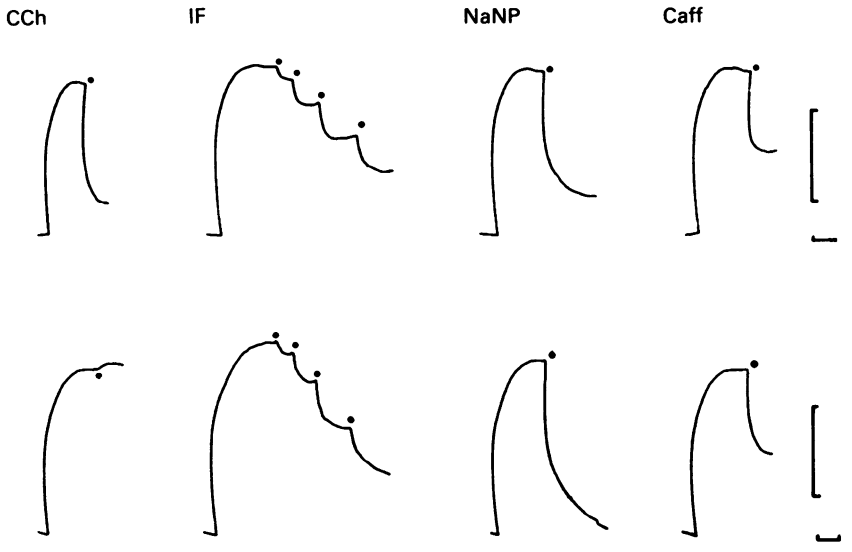

Figure 1 Representative records illustrating the relaxant effects of carbachol (CCh, 3 μM), inhibitory factor from the bovine retractor penis (IF, 10, 25, 50 and 100 μl), sodium nitroprusside (NaNP, 30 nM) and caffeine (Caff, 1 mM) on noradrenaline-induced contractions of rabbit aortic strips. The strips in the upper records were unrubbed, i.e. with intact endothelium, those in the lower records were rubbed to remove the endothelium. Calibration bars 1 g and 5 min.

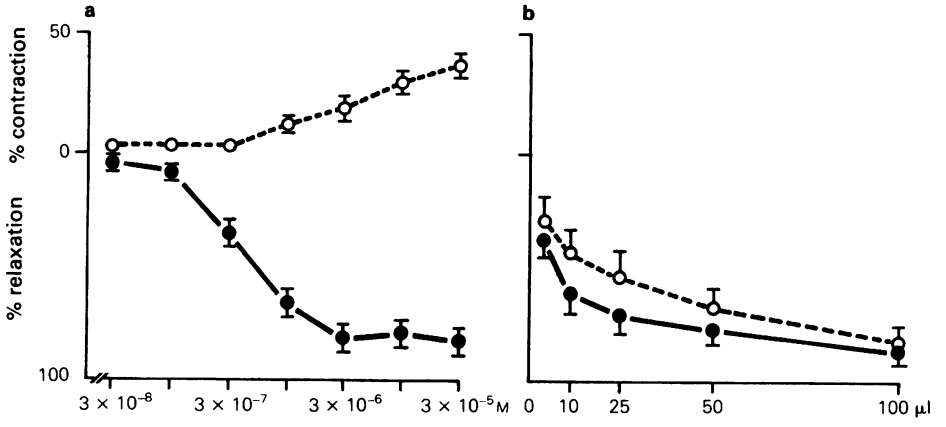

Figure 2 Cumulative dose-response curves for the relaxant effect of (a) carbachol and (b) the inhibitory factor from the bovine retractor penis in rabbit isolated aortic strips pre-contracted with noradrenaline: (●) unrubbed strips with intact endothelium; (○) strips from which the endothelium had been removed by rubbing. Each point is the mean of between 10 and 15 observations for carbachol and 8 observations for the inhibitory factor. The bars are ± the s.e.mean. The responses are the increases or decreases in tone expressed as a percentage of the noradrenaline-induced contraction. In these experiments 1 ml of the solution of inhibitory factor corresponds to 3 g wet weight of muscle.

cyclase (Bowman & Drummond, 1984). Sodium nitorprusside relaxation is also believed to be due to guanylate cyclase activation (Arnold *et al.*, 1977). We, therefore, examined the effect of nordihydroguaiaretic acid on the relaxant effect of sodium nitroprusside. Nordihydroguaiaretic acid potentiated relaxation by sodium nitroprusside (Figure 4) just as it potentiated relaxation by the inhibitory factor; the vehicle DMSO was without significant effect.

The effect of methylene blue

The EDRF is dependent for its action on vascular smooth muscle on the activation of guanylate cyclase (Rapoport & Murad, 1983a; Diamond & Chu, 1983). Inhibition of the BRP by the inhibitory factor and by the inhibitory nerves also appear to be linked to guanylate cyclase activation (Bowman & Drummond, 1984). We, therefore, tested the effect of methylene

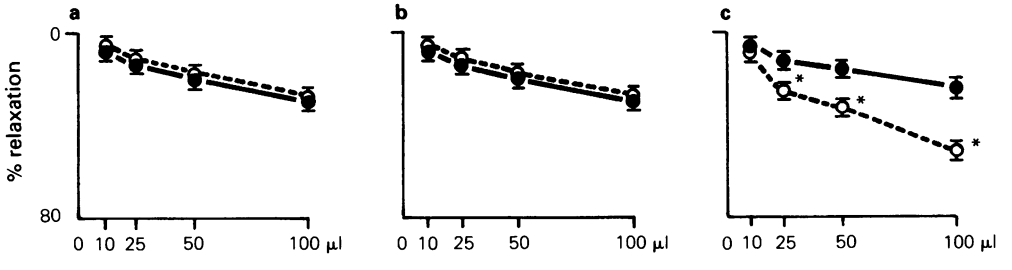

Figure 3 The effect of (a) quinacrine 10 µM, (b) flurbiprofen 15 µM and (c) nordihydroguaiaretic acid 25 µM on the relaxation of noradrenaline-induced tone in the unrubbed rabbit aortic strip by the inhibitory factor from the bovine retractor penis. In each panel (●) = the control responses; (○) = responses in the presence of the appropriate drug. Each point is the mean of five observations with s.e.mean indicated by bars; an asterisk indicates significant differences at the $P < 0.01$ level. In these experiments 1 ml of the solution of inhibitory factor corresponds to 1 g wet weight of muscle.

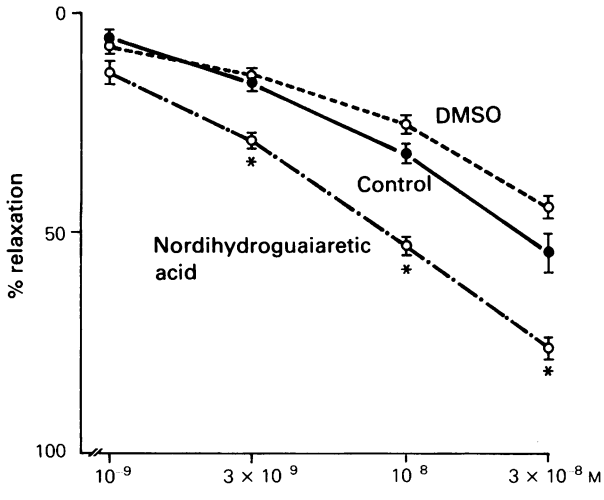

Figure 4 The effect of nordihydroguaiaretic acid, 25 μM, and its vehicle dimethylsulphoxide (DMSO) 0.1%, on the relaxant effect of sodium nitroprusside in unrubbed rabbit aortic strips pre-contracted with noradrenaline. Each drug was added to the organ bath 20 min before contraction by noradrenaline. Each point represents the mean of four observations with s.e.mean indicated by bars; the asterisk denotes significant difference between means at the $P < 0.01$ level.

blue, a drug reported to inhibit guanylate cyclase (Ignarro *et al.*, 1981; Kukovetz *et al.*, 1982) on the relaxant effect of the inhibitory factor on arterial smooth muscle. As controls we examined the effects of methylene blue on relaxation produced by carbachol, sodium nitroprusside and caffeine. At 10 μM methylene blue the carbachol-induced relaxations were almost completely abolished with no effects on relaxation by the inhibitory factor, sodium nitroprusside or caffeine (Figure 5a). Increasing the methylene blue concentration to 30 μM completely abolished the inhibitory effect of carbachol, but, in addition, almost completely abolished the relaxation by the inhibitory factor and significantly reduced relaxation by sodium nitroprusside (Figure 5b). Surprisingly, the relaxant effect of caffeine was significantly increased by this dose of methylene blue (Figure 5b).

Discussion

Relaxation of arterial smooth muscle preparations by carbachol and several other agents is characteristically dependent upon the presence of the endothelium. The present results show that the inhibitory factor derived from the BRP does not produce arterial relaxation indirectly by releasing an endothelium-dependent relaxing factor. If the action of the inhibitory factor is direct on the smooth muscle, the possibility that the inhibitory factor and EDRF are identical is raised. However, there are other reasons, such as the need for acid activation of inhibitory factor before biological activity is obtained, which are against such an identity. The results reported here with inhibitors of eicosanoid metabolite formation are also, at first sight, against such a possibility. While quinacrine and nordihydroguaiaretic acid abolish the relaxant effect of carbachol, they did not reduce that of the inhibitory factor. This argument is valid only on the assumption that the enzymes inhibited by these drugs are required for the muscle response to the EDRF. If they are involved only in its formation and release than their inhibition would reduce neither the response to EDRF nor the inhibitory factor.

The effects of methylene blue, a drug reported to inhibit guanylate cyclase (Ignarro *et al.*, 1981; Kukovetz *et al.*, 1982) appear more helpful. Methylene blue (10 μM) almost completely abolished the relaxation induced by carbachol whilst that of the inhibitory factor was unaffected. Unfortunately, quite a small increase in the dose of methylene blue, to 30 μM, results in an almost complete abolition of the relaxant action of the inhibitory factor from the BRP. It may be that the different sensitivity of carbachol-induced and inhibitory factor-induced relaxation to methylene blue does indicate a different mode of action but it is just as likely that the difference lies in the site of action of inhibition. Though no information is available on the thickness of smooth muscle acted on by the EDRF, given its origin in endothelial cells and its short half life (Forstermann *et al.*, 1985), it is probable that this is confined to a few muscle layers closest to the intima. The inhibitory factor on the other hand may act throughout the muscle layers. If these muscle layers vary in sensitivity to methylene blue then this could account for the findings. It is also possible that the guanylate cyclase acted on by the different stimuli are different. If carbachol acted on a guanylate cyclase mainly localized in the membrane, and inhibitory factor acted on guanylate cyclase in both membrane and cytoplasm and sodium nitroprusside mainly on cytoplasmic guanylate cyclase, this would provide another explanation. From the experiments reported in this paper it is, therefore, not possible to distinguish with certainty between the EDRF and the inhibitory factor from the BRP.

The increased relaxant effect of both the inhibitory factor from the BRP and sodium nitroprusside in the presence of nordihydroguaiaretic acid was unexpected. It appears to be selective at least to the extent that caffeine is not similarly potentiated. Since both the inhibitory factor and sodium nitroprusside (Arnold *et al.*, 1977) appear to share a common mechanism of relaxation by activating guanylate cyclase it may be

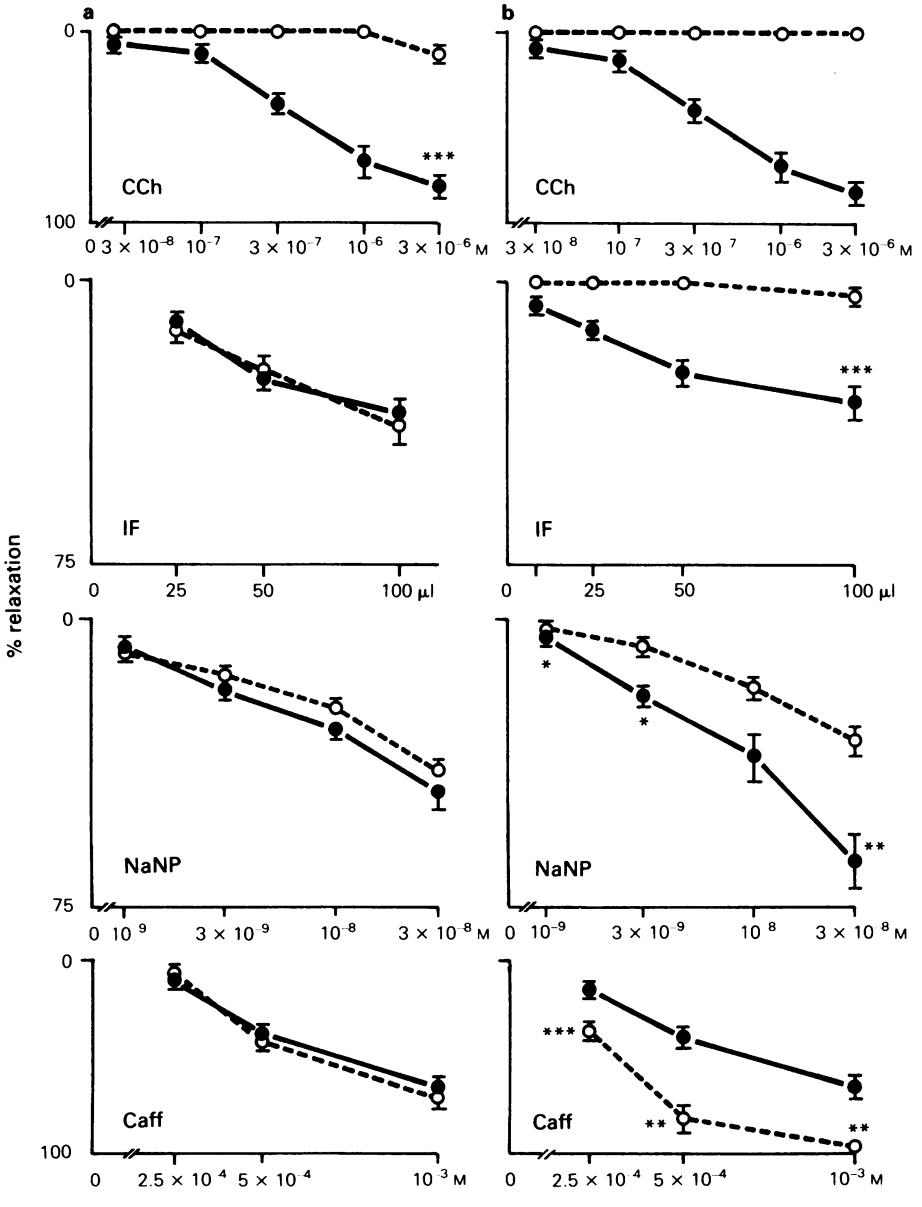

Figure 5 The effect of methylene blue (a) 10 μM and (b) 30 μM on the cumulative dose-response curves for relaxation by carbachol (CCh), inhibitory factor from the bovine retractor penis (IF), sodium nitroprusside (NaNP) and caffeine (Caff) in the unrubbed rabbit aortic strip. The strips were pre-contracted with noradrenaline. Each point is the mean of 5 to 6 observations with s.e.mean indicated by bars; statistically significant differences are shown by * $P < 0.05$; ** $P < 0.02$; *** $P < 0.01$. In these experiments 1 ml of the inhibitory factor solution corresponds to 1 g wet weight of muscle.

that nordihydroguaiaretic acid makes this more effective possibly by preventing the formation of some lipoxygenase induced by noradrenaline and acting to inhibit guanylate cyclase. We have, however, no evidence for this nor an explanation for the unexpected potentiation of caffeine relaxation by methylene blue (30 μM). Experiments in progress to measure changes in guanylate cyclase activity by these drugs may throw light on these unexplained effects.

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